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Authors

Hillman, Noah H
Gisslen, Tate
Polglase, Graeme R
et al.

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Ventilation-Induced Increases in EGFR Ligand mRNA Are Not Altered by Intra-Amniotic LPS or Ureaplasma in Preterm Lambs

Noah H. Hillman^{1,2*}, Tate Gisslen¹, Graeme R. Polglase^{3,4}, Suhas G. Kallapur^{2,3}, Alan H. Jobe^{2,3}

1 Division of Neonatology, Saint Louis University, Saint Louis, Missouri, United States of America, **2** Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, Ohio, United States of America, **3** School of Women and Infants' Health, University of Western Australia Perth, WA, Australia, **4** The Ritchie Centre, Monash Institute of Medical Research, Monash University, Melbourne, VIC, Australia

Abstract

Chorioamnionitis and mechanical ventilation are associated with bronchopulmonary dysplasia (BPD) in preterm infants. Mechanical ventilation at birth activates both inflammatory and acute phase responses. These responses can be partially modulated by previous exposure to intra-amniotic (IA) LPS or Ureaplasma parvum (UP). Epidermal growth factor receptor (EGFR) ligands participate in lung development, and angiotensin converting enzyme (ACE) 1 and ACE2 contribute to lung inflammation. We asked whether brief mechanical ventilation at birth altered EGFR and ACE pathways and if antenatal exposure to IA LPS or UP could modulate these effects. Ewes were exposed to IA injections of UP, LPS or saline multiple days prior to preterm delivery at 85% gestation. Lambs were either immediately euthanized or mechanically ventilated for 2 to 3 hr. IA UP and LPS cause modest changes in the EGFR ligands amphiregulin (AREG), epiregulin (EREG), heparin binding epidermal growth factor (HB-EGF), and betacellulin (BTC) mRNA expression. Mechanical ventilation greatly increased mRNA expression of AREG, EREG, and HB-EGF, with no additional increases resulting from IA LPS or UP. With ventilation AREG and EREG mRNA localized to cells in terminal airspace. EGFR mRNA also increased with mechanical ventilation. IA UP and LPS decreased ACE1 mRNA and increased ACE2 mRNA, resulting in a 4 fold change in the ACE1/ACE2 ratio. Mechanical ventilation with large tidal volumes increased both ACE1 and ACE2 expression. The alterations seen in ACE with IA exposures and EGFR pathways with mechanical ventilation may contribute to the development of BPD in preterm infants.

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* E-mail: Hillman@slu.edu

Introduction

Mechanical ventilation at birth can easily injure the preterm lung and activate a systemic acute phase response [1,2]. Although this initial lung inflammation may contribute to the development of bronchopulmonary dysplasia (BPD) in very low birth weight infants (VLBW), other molecular pathways are also activated by mechanical ventilation [1]. Many of these pathways contribute to the later stages of lung development and perhaps repair of the initial ventilation induced injury. Even small alterations in expression may contribute to the alveolar simplification seen in infants with BPD [3]. Although clinicians have tried to decrease exposure to mechanical ventilation to decrease BPD, BPD rates have not declined substantially with the introduction of less invasive mechanical ventilation [4-6].

The combination of antenatal fetal exposure to chorioamnionitis and post-delivery mechanical ventilation was associated with an increased risk of BPD [7]. This is an interesting paradigm because antenatal exposure to intra-amniotic (IA) E. coli lipopolysaccharide (LPS) induces lung maturation in sheep, and clinical chorioamnionitis can decrease respiratory distress syndrome in infants [7,8]. Nonetheless, preterm infants exposed to

chorioamnionitis accompanied by fetal inflammatory response have a poor response to surfactant treatment and increased BPD [9]. Exposure to IA Ureaplasma parvum (UP) causes a milder inflammatory response than LPS, less consistent lung maturation, and reduced lung injury after mechanical ventilation [10,11]. We previously demonstrated that IA exposure to LPS or UP modulates subsequent exposures to toll-like receptor agonists [8,12,13]. The development of BPD is likely promoted by a combination of multiple prenatal and antenatal exposures. Understanding the molecular pathways activated during mechanical ventilation at birth in the setting of chorioamnionitis should provide information about regulatory pathways that are activated or suppressed by fetal and early neonatal exposures.

Alterations in growth factors and metabolic pathways within the lung have been clinically associated with both BPD and lung disease in children [14,15]. Our preliminary analysis of mRNA sequencing of mechanically ventilated lambs demonstrated possible changes in two important pathways in the lung; 1) epidermal growth factor receptor (EGFR) and 2) angiotensin converting enzymes (ACE). EGFR regulates airway branching and alveolar maturation, and mutations in EGFR receptor are found in some forms of non-small cell lung cancer [16,17]. The EGFR ligand

amphiregulin (AREG) increases with mechanical ventilation [18]. EGFR can also be triggered by multiple other ligands, including epiregulin (EREG), heparin binding-epidermal growth factor (HB-EGF), and betacellulin (BTC) [17]. ACE 1 and ACE2 are enzymes produced in the lung that can modulate lung inflammation, and ACE1 gene polymorphisms may affect severity of lung diseases [19,20]. Using tissue from previous preterm sheep models [10–12], we examined whether antenatal exposure to LPS or UP alters gene expression for EGFR, EGFR ligands and ACE in the lung. We further explored the effects of mechanical ventilation on expression of these genes, with or without antenatal LPS or UP exposures, in the setting of normal (7 mL/kg) and large (15 mL/kg) tidal volume ventilation.

Methods

The investigations were approved by the Animal Ethics Committees of the University of Western Australia and Cincinnati Children's Hospital Medical Center. Lung physiology and inflammatory responses of these animals were described previously [10–12]. The role of chorioamnionitis on EGFR and ACE pathways was tested using animals exposed to LPS or UP compared to unventilated controls [10,12]. The effects of mechanical ventilation were evaluated in animals with or without intra-amniotic inflammation from LPS or UP to look for additive/protective effects [11,12]. Mechanically ventilated, ureaplasma-exposed or saline-exposed animals were used to determine if the V_T used during the initial 15 minutes of ventilation affected the response of the pathways [11]. The intervention groups are described below and summarized in Table 1.

LPS groups

Time-mated ewes were randomly assigned to receive 2 ml ultrasound guided IA injection of 10 mg *Escherichia coli* LPS O55:B5 (Sigma-Aldrich, St. Louis, MO) or 2 ml saline at both 123 d and 128 d gestational age before operative delivery at 130 d gestation (term is 150 d) [21]. Surfactant treatments (100 mg/kg Curosurf, a gift from Chiesi Pharma, Parma, Italy) were given to normalize surfactant pools prior to ventilation [22]. The lambs were mechanically ventilated with volume guarantee targeting a

tidal volume (V_T) of 7 mL/kg (Dräger Babylog 8000+, Lübeck, Germany) for 120 minutes [12]. Lambs receiving the two doses of LPS but no ventilation (LPS group) and lambs receiving no IA injection or ventilation (UVC) were used as comparison groups.

Ureaplasma groups

In ureaplasma exposed lambs (UP group), lambs received 2 ml saline IA containing *Ureaplasma parvum* serovar 3 (2×10^7 CFU) at 110 d GA and were delivered at 124 d GA, immediately euthanized, and tissues collected [10]. Ewes for ventilation studies had IA UP at 55 d gestation [23]. Fetuses in both 55 d IA UP and unexposed groups were randomized at 128 ± 1 d gestation to one of two ventilator strategies: 1) surfactant treatment and then mechanical ventilation with a V_T of 7 mL/kg for 3 hours or 2) mechanical ventilation with V_T escalating to 15 mL/kg (V_{T15}) at 15 min then surfactant treatment and ventilation for 2 hr 45 min at 7 mL/kg. Non-ventilated controls (UVC) and non-ventilated UP (UP UVC) were euthanized prior to delivery and were sampled immediately.

Quantitative RT-PCR. mRNA was extracted from lung tissue with TRIzol (Invitrogen, USA) and DNase treated. cDNA was produced using Verso cDNA kit (Thermoscientific, UK). Custom Taqman gene primers (Applied Biosystems, USA) were designed from ovine sequences for Amphiregulin (AREG), Epiregulin (EREG), Heparin binding-epidermal growth factor (HB-EGF), Betacellulin (BTC), Epidermal growth factor receptor (EGFR), Angiotensin converting enzyme 1 (ACE 1), ACE2, Hepatic growth factor and Midkine. Quantitative RT-PCR was performed with a 7300 RT-PCR machine and software (Applied Biosystems, USA). 18S primers were used for internal loading controls, and results are reported as fold increase over mean for unventilated control animals.

In situ Hybridization. In situ localization of AREG and EREG mRNA used digoxigenin-labeled anti-sense and sense sheep riboprobes synthesized from cDNA templates using DIG RNA labeling kits (Roche, USA). The sections were pre-treated with paraformaldehyde, proteinase K, and hybridized overnight. Sections were formamide washed, RNase A treated, then blocked with 10% horse serum. Following incubation overnight with anti-

Table 1. Groups, intra-amniotic (IA) exposures, gestational age and ventilation variables.

LPS Groups	IA Exposure	GA at delivery	N	V_T 15 min mL/kg	V_T 105 min mL/kg
Saline Controls (a)	Saline 2 & 7d	130d	5	—	—
IA LPS (a)	LPS 2d & 7d	130d	5	—	—
IA LPS+Vent (a)	LPS 2& 7d	130d	5	6.8 ± 0.2	6.7 ± 0.6
IA Saline+Vent (a)	Saline 2 & 7d	130d	5	6.6 ± 0.2	5.8 ± 0.4
Ureaplasma Groups	IA Exposure		N	V_T 15 min mL/kg	V_T 165 min mL/kg
Unventilated controls (b)	none	124d	7	—	—
IA Ureaplasma (b)	14d UP	124d	5	—	—
UP UVC Controls (c)	none	128d	6	—	—
IA UP+Vent 7mL/kg (c)	55d UP	128d	5	6.8 ± 1.1	8.2 ± 1.2
Vent 15mL/kg (c)	none	128d	5	13.2 ± 1.8	7.7 ± 0.7
IA UP+Vent 15mL/kg (c)	55d UP	128d	5	14.2 ± 1.9	7.4 ± 0.5

(a) Gisslen *et al.* Innate immunity 2013 [12], (b) Collins *et al.* AJP-Lung 2010 [10], (c) Polglase *et al.* Pediatr Res 2010[11]
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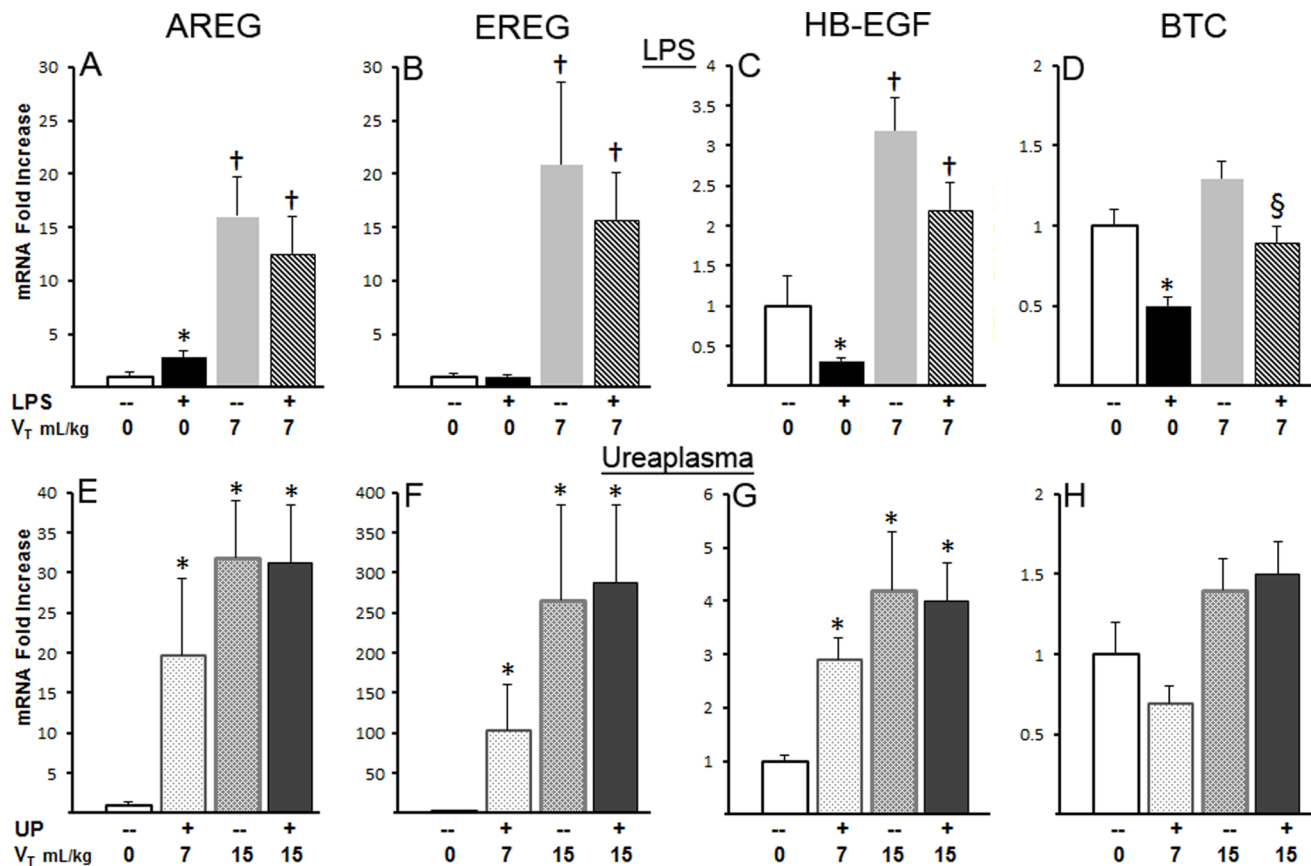


Figure 1. EGFR ligand mRNA response in lung to IA LPS, IA Ureaplasma, and mechanical ventilation. (A–D) mRNA response to IA-LPS and mechanical ventilation at 7 mL/kg (V₇). (A) Amphiregulin (AREG) increases with LPS exposure with further increase with V₇. (B) The Epregrulin (EREG) increase with V₇ is not altered by LPS. (C) Heparin binding epithelial growth factor (HB-EGF) decreases with LPS exposure, but increases with V₇. (D) Betacellulin (BTC) decreases with LPS and this decrease with LPS remains during V₇. (E–H) mRNA response to IA-UP, UP+V₇, mechanical ventilation at 15 mL/kg (V₁₅) and V₁₅+UP. (E) AREG and (F) EREG increase with V₇ and V₁₅. (G) HB-EGF increases with V₇ and V₁₅. (H) BTC had no change with UP or ventilation. * $p < 0.05$ vs unventilated, unexposed controls (UVC), † $p < 0.05$ vs UVC and IA-LPS, § $p < 0.05$ vs UVC and V₇ without LPS

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Digoxigenin antibody (Roche, USA), the slides were developed with NBT-BCIP (Roche, USA).

Data Analysis. Results are shown as mean (SEM). Significance was accepted as $p < 0.05$ using Student's t-test or Mann-Whitney non-parametric (InStat GraphPad, USA).

Results

The lambs were similar in size, gestational age, and ventilation variables across the groups, as previously reported [10–12].

EGFR Ligand and EGFR mRNA expression

Intra-amniotic exposure to LPS caused a small but significant increase in AREG mRNA, and decreases in HB-EGF and BTC ($p < 0.05$) (Figure 1A, C, D). Mechanical ventilation strongly induced mRNA for the EGFR ligands AREG, EREG, and HB-EGF, but had no effect on BTC (Figure 1). A similar magnitude increase in AREG (12 to 17 fold), EREG (15 to 50 fold), and HB-EGF (2 to 3 fold) was measured for all animals ventilated at 7 mL/kg, regardless of previous exposure to intra-amniotic LPS or UP. Animals ventilated with 15 mL/kg for 15 minutes then 7 mL/kg for 105 min had generally higher induction of AREG, EREG, HB-EGF with no effect of prior UP exposure (Figure E–G). The small decrease in BTC with LPS exposure was maintained with

ventilation compared with animals not receiving IA LPS (Figure 1D). Intra-uterine exposure to Ureaplasma caused a similar small decrease in HB-EGF (Table 2).

In situ localization of EREG demonstrated increased mRNA signal in domed cells within the distal lung (Figure 2C, Insert). Control lambs and lambs exposed to LPS did not demonstrate in situ signal (Figure 2A,B). AREG mRNA signal is also increased in distal domed cells with ventilation (Figure 2F), and not found in similar appearing cells in either control (Figure 2D) or LPS exposed animals (Figure 2E). Cells with AREG and EREG mRNA signal did not co-stain with smooth muscle actin (data not shown), and are located in appropriate location for type II pneumocytes.

IA LPS exposure caused a 30% decrease in EGFR mRNA ($p < 0.05$) (Table 3), whereas IA UP had no effect (Table 2). Mechanical ventilation, with or without IA LPS or UP, caused an average 1.5 ± 0.1 fold increase in EGFR mRNA ($p < 0.05$ in each ventilated groups vs. controls) (Table 3).

Angiotensin Converting Enzyme mRNA

Angiotensin converting enzyme (ACE) is produced in the lung and the balance between ACE 1 and ACE2 may contribute to lung injury or repair [19]. IA LPS exposure decreased in ACE1 mRNA (Figure 3A) and increased ACE2 mRNA (Figure 3B), resulting in a 4-fold increase in the ratio of ACE2/ACE1

Table 2. mRNA Fold increase over controls with 14 Day Ureaplasma exposure.

AREG	EREG	HB-EGF	BTC	EGFR	ACE1	ACE2	ACE1/2	Midkine	HGF
1.1±0.4	2.1±0.5	0.7±0.03*	1.0±0.2	1.0±0.1	0.7±0.03*	1.5±0.2*	2.2±0.2*	1.2±0.1	0.9±0.1

* p<0.05 vs saline controls.

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(Figure 3C). 14 day exposure to UP caused a similar decrease in ACE1 mRNA and a small increase in ACE2 mRNA, which increased the ACE2/ACE1 ratio 2 fold (Table 2). Mechanical ventilation with 7 mL/kg did not alter ACE mRNA (Figure 3A–C). Ventilation with 15 mL/kg increased ACE1 mRNA (1.5 ± 0.1 fold), ACE2 mRNA (2.3 ± 0.2 fold), and the ratio of ACE2/ACE1 (1.6 ± 0.3 fold) versus unventilated controls ($p < 0.05$ for all). Midkine mRNA decreased somewhat with ventilation of LPS exposed lambs (Table 3). There were no changes in the mRNA for midkine or hepatic growth factor with 14 d UP (Table 2).

Discussion

Chorioamnionitis and mechanical ventilation are associated with an increased risk of BPD in VLBW infants [7]. Growth factors important for lung development (EGFR and its ligands) and ACE1, ACE2 in the lung were altered by either antenatal inflammation or mechanical ventilation. We demonstrated a striking increase in the mRNA for EGFR ligands with mechanical ventilation at birth, especially with a V_T of 15 mL/kg, and a subsequent 1.5 fold increase in EGFR mRNA. Baseline EGFR mRNA ligands were modestly changed with antenatal exposure to LPS or UP. In contrast to the EGFR ligands, ACE1 and ACE2 were altered by antenatal exposure to LP or UP, but minimally changed by mechanical ventilation. These findings identify several molecular pathways activated at birth that may contribute to the development of BPD.

One of our primary findings is the striking up-regulation of multiple EGFR ligands during mechanical ventilation of preterm lambs. Our observation that AREG increased with mechanical ventilation is consistent with gene arrays that demonstrate increased AREG in ventilated adult mice, but not to the degree seen in these preterm lambs [18]. In isolated mouse lungs, ventilation with a large tidal volume increased AREG mRNA and protein compared to lungs receiving more moderate tidal volumes [18]. The AREG protein was increased in the peripheral lung, though exact cell type was not determined [18]. In contrast to our antenatal exposure, postnatal LPS exposure in mice further increased the mRNA values when lungs were ventilated with high tidal volumes and LPS exposure [18]. EGFR ligands can also be excreted by pulmonary cells in response to stimulation; AREG is secreted from human pulmonary epithelial cells with exposure to cigarette smoke or particulate matter [24]. Mechanical compression of human bronchial epithelial cells (NHBE cells) caused the shedding of HB-EGF into the extracellular fluid and an autocrine activation of the EGFR receptor [25]. Mechanical stretch also released HB-EGF, EGF, AREG, and BTC [26]. The trends for further increases in mRNA for EGFR ligands in lambs receiving more injurious ventilation (15 mL/kg) corresponded with our previously reported increased inflammatory markers with higher V_T ventilation in the preterm lung [11]. This result is consistent with *in vitro* studies of stretched type II cells, where increased duration of stretch caused larger mRNA induction [27]. Our findings and previous studies demonstrate an increase in multiple EGFR ligands and EGFR mRNA in response to mechanical

ventilation, and the intensity of the response corresponds to the degree of mechanical stretch.

The production of mRNA for EGFR ligands by domed-cells in the peripheral lung with mechanical ventilation may have a large effect on the developing alveoli and could contribute to the development of BPD. EGF has an important role in lung development and cellular differentiation. EGFR deficient mice have decreased airway branching, decreased alveolarization, type II cell immaturity, and respiratory distress at birth [28]. Similarly, pregnant rats given anti-EGF antibodies deliver pups with respiratory distress and decreased lung size [29]. Fetal administration of EGF to preterm rabbits caused increased lung maturation and number of type II alveolar epithelial cells [30]. HB-EGF promotes type II epithelial differentiation with increased mRNA for surfactant proteins B and C, whereas type II cells incubated with AREG, EREG or BTC did not have increased surfactant mRNA [26]. Over-expression of EGFR can also cause injury, as EGF stimulation in type II cells can lead to alveolar tumors and EGFR gene mutations are found in about 10% of non-small cell lung tumors [31]. AREG also participates in pulmonary fibrosis by modulation of TGF- α [32], and EGFR inhibitors can decrease pulmonary fibrosis [33]. Alterations of EGF, EGFR, and TGF- α were reported in lungs from infants with BPD [34]. Increased expression of AREG also contributes to the mucus cell metaplasia seen in naphthalene-lung injury [35]. In contrast, AREG given intra-peritoneally to mice decreased the lung

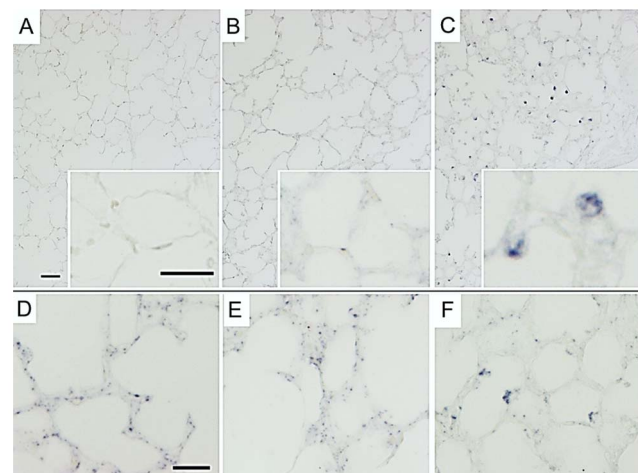


Figure 2. In situ localization of EREG and AREG with LPS exposure and mechanical ventilation in lung. (A–C) EREG mRNA was not found in (A) unventilated controls or (B) IA LPS exposed lungs, but dramatically increased in peripheral lungs of lambs receiving (C) mechanical ventilation. EREG mRNA is localized to dome-shaped cells in peripheral lung (C, Insert). (D–F) AREG mRNA is found scattered in the peripheral lung of (D) Unventilated controls with a mild increase with (E) IA LPS exposure. (F) Mechanical ventilation increases the mRNA signal in similar cells to EREG. Scale bar = 50 μ m
doi:10.1371/journal.pone.0096087.g002

Table 3. EGFR and Midkine mRNA fold changes.

Groups	EGFR	Midkine
Controls	1.0±0.1	1.0±0.1
LPS	0.7±0.04*	0.9±0.1
Vent 7 mL/kg	1.3±0.1 [†]	0.7±0.1
LPS+Vent 7 mL/kg	1.4±0.1 [†]	0.5±0.1 [†]
Vent 15 mL/kg	1.6±0.2*	1.1±0.1
UP+Vent 15 mL/kg	1.8±0.3*	0.8±0.1

*p<0.05 vs Controls; [†]p<0.05 vs Controls and LPS alone.

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inflammation and fibrosis caused by bleomycin [36]. It is presently unclear whether the production of EGFR ligands during mechanical ventilation is a protective or harmful response of the preterm lung.

Although we did not measure a large effect of antenatal exposure to LPS or Ureaplasma on EGFR ligand expression, IA exposure can alter pro-inflammatory pathways in fetal sheep [8,12,13]. Long term exposure to UP decreased the response to IA LPS, demonstrating fetal cross-tolerance to toll-like receptors [13]. Fetal sheep develop tolerance when exposed to multiple doses of E. Coli LPS [8]. IA LPS also causes a partial tolerance to IV LPS in ventilated preterm sheep [12]. Since IA LPS also matures the macrophages within the lungs and alters their response subsequent stimulation, the lack of large changes in EGFR ligands with IA LPS or Ureaplasma suggests these cells are likely not the primary source of EGFR activation [37]. The additive effects of chorioamnionitis and mechanical ventilation [7] are likely not due to changes in EGFR or ligands.

We demonstrated a decrease in ACE1 mRNA and an increase in ACE2 mRNA with exposure to intra-amniotic LPS and UP. There was little additional effect on the ACE ratio with short-term mechanical ventilation at 7 mL/kg, though larger V_T ventilation increased both ACE1 and ACE2 mRNA. The change in the ACE ratio from the antenatal exposure may also contribute to BPD by effects on lung inflammation. ACE1 cleaves angiotensin I to angiotensin II, which is a potent activator of a variety of receptors, and ACE2 can inactivate angiotensin II and convert it to

Angiotensin 1–7. ACE1 activation causes lung inflammation by activation of NF-κB [19], and ACE enzyme activity is elevated in the airway fluid of patients with ARDS [15]. ACE1 inhibitors and angiotensin II receptor antagonists decrease lung inflammation and fibrin deposition in multiple animal models of ARDS [19,38]. There is speculation that polymorphisms in ACE gene contribute to mortality from ARDS [20]. In small clinical studies, ACE gene polymorphisms were not associated with BPD or RDS [39,40]. ACE2 protected the lung against injury in animal models of ARDS [41], and supplementation of recombinant ACE2 decreased lung fibrosis in rats exposed to bleomycin-induced lung injury [42]. ACE1 and ACE2 enzyme activity counter-balance each other within the lung, and the ratio of the enzymes (ACE1/ACE2) correlates with the extent of lung injury [43]. In rats exposed to intratracheal LPS 24 hours prior to ventilation, ACE1 increases and ACE2 decreases, but only in the ventilated animals [43]. Correction of the imbalance of ACE2/ACE1 activity with either Angiotensin 1–7 or blockade of ACE1 activity with losartan decreased the lung injury [43]. We found an opposite response in the ACE2/ACE1 ratio, likely due to antenatal LPS exposure in preterm lambs. The components of the renin-angiotensin system are present in the mouse lung at the pseudoglandular stage of lung development (E13.5) and angiotensin II contributes to increased lung branching in lung explants [44]. ACE inhibitors given to neonatal rats cause larger airspaces, thinner alveolar septum, and lower surface tension in the BAL, suggesting a role of the angiotensin system in lung development [45]. Although modest,

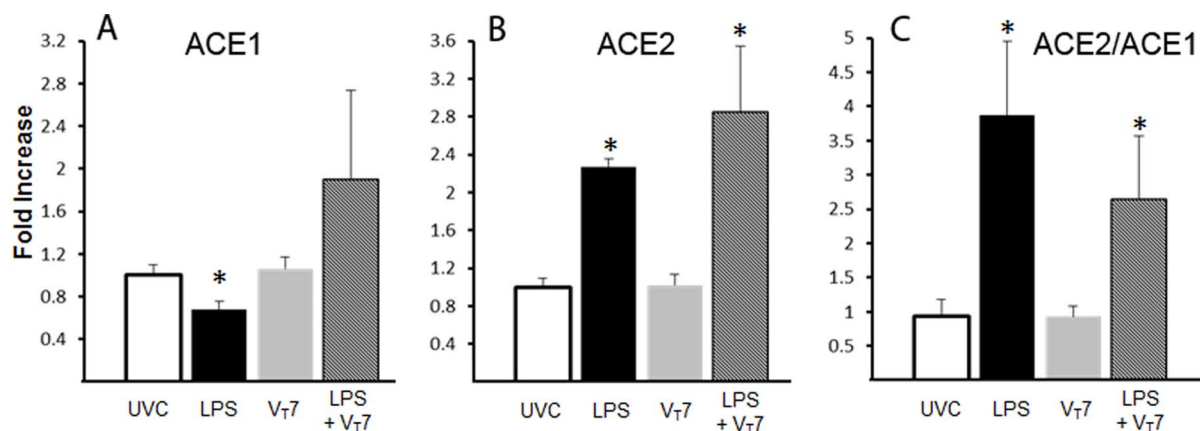


Figure 3. Angiotensin converting enzyme (ACE) 1 and 2 mRNA in lung with exposure to LPS, UP and mechanical ventilation. (A–C) mRNA values for unventilated controls (UVC), IA-LPS, and mechanical ventilation at 7 mL/kg (V_T7). (A) ACE1 was decreased by LPS but unaffected by V_T7. (B) ACE2 was increased by LPS and LPS+V_T7. (C) ACE2/ACE1 mRNA ratio increased with LPS and LPS+V_T7. * p<0.05 vs UVC.

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the two to four-fold change in the ACE2/ACE1 ratio caused by antenatal exposure to LPS or UP could contribute to in the alveolar simplification of BPD.

One of the limitations of the study is the assumption that mRNA is translated into proteins and have a physiologic response. The consistency and magnitude of mRNA increases for multiple ligands across 3 animal groups, and subsequent increase in mRNA for the receptor suggest the mRNA is translated. The mRNA increases are represented as fold increases over control values, but not as absolute values of mRNA. Although only a rough estimate of relative abundance, the Ct values were 5 cycles earlier in HB-EGF and EGFR mRNA than for AREG and EREG, suggesting these mRNA are considerably more abundant. The increases in AREG and EREG mRNA from control baseline were similar to those for the cytokines previously reported [11,12]. A similar 5 cycle difference in Ct is also seen between ACE1 and ACE2 mRNA, suggesting ACE1 is more prevalent in the developing lung. Interpretation of Ct values must be done with some caution, as the relative efficiency of each PCR probe may contribute to differences, thus fold change over controls was reported.

Lung and systemic inflammation are associated with both the initiation of ventilation of preterm lambs and chorioamnionitis,

and likely contribute to BPD [10,17,40]. We now demonstrate two additional molecular pathways that add more complexity to understanding the pathogenesis of BPD. Mechanical ventilation of preterm lambs caused an increase in EGFR ligands which can influence cell differentiation, but the EGFR ligands were not modulated by LPS or UP exposure. Conversely, the ratio of ACE1 to ACE2 was altered by prenatal exposure to LPS or UP but not altered by short term mechanical ventilation. The link between chorioamnionitis and mechanical ventilation may not be an additive effect on a specific molecular pathway, but a combination of effects on multiple pathways. Understanding the additional molecular pathways activated in premature infants by mechanical ventilation may provide new therapeutic treatment for infants with BPD.

Author Contributions

Conceived and designed the experiments: NHH SGK AHJ. Performed the experiments: NHH TG GRP SGK AHJ. Analyzed the data: NHH AHJ. Contributed reagents/materials/analysis tools: NHH SGK AHJ. Wrote the paper: NHH TG GRP SGK AHJ.

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